Is there any relationship between PSA and increased peripheral $CD4^+CD25^{high}FOX3^+$ Treg in prostate cancer patients?

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Abstract

Introduction: The aims of this study were first, to determine whether peripheral levels of $CD4^+CD25^{high}Foxp3^+$ regulatory T cells (Treg) are elevated in Prostate Cancer (PCa) patients, and second, to determine the direct correlation between peripheral Treg and total serum Prostate Specific Antigen (PSA) levels in these patients.

Methods: Peripheral Blood Mononuclear Cells from 56 subjects undergoing diagnostic prostate biopsies (PSA ≥ 2.5 ng/ml) were analyzed for Treg numbers. The association between the peripheral Treg and serum PSA values was first determined in the entire population, including people with no prostate pathology and PCa and Benign Prostate Hyperplasia (BPH) patients, and second, in nine PCa patients before and after curative prostatectomy.

Results: This project was performed in Akdeniz University immunology laboratory and urology out patient clinic from 2008 to 2010. Peripheral Treg frequencies were significantly increased in PCa patients (n = 19, 3.23 ± 1.59) compared with BPH patients (n = 27, 1.66 ± 0.80) and healthy subjects (n = 10, 1.08 ± 0.43) ($p < 0.01$). The percentage of Treg in BPH patients was also significantly higher than that of healthy subjects ($p < 0.01$). Importantly, the increase in BPH and PCa patients paralleled the elevation in total serum PSA levels, demonstrating a strong positive correlation ($r = 0.75$; $p < 0.01$).

Conclusion: These results demonstrate that peripheral Treg densities are correlated with PSA in BPH and PCa patients, suggesting that PSA may have a role in Treg induction and/or maintenance in Treg in these people.

Keywords: $CD4^+CD25^{high}Foxp3^+$ Regulatory T cells (Treg), prostate cancer, benign prostate hyperplasia, PSA, TAA

Introduction

Prostate cancer (PCa) is the most commonly diagnosed cancer among men in the world (1). Approximately two thirds of PCa cases are confined to the prostate and can be treated by radical prostate removal or radiotherapy (2). In addition, approximately 25 to 55% of treated, locally confined tumors reappear within 10 years and may progress as either a local recurrence or distant metastases (3). In the quest for effective prevention and treatment modalities for metastatic PCa (4), immunotherapy attempts using several different methods have shown very limited success (5-9). Various immune evasion mechanisms, such as defects in antigen presentation, secretion of immunosuppressive agents by the tumor cells, and T cell receptor defects, are thought to limit the success of these trials. Recently, natural regulatory T cells ($CD4^+CD25^{high}$; Treg), one of the key regulators of self tolerance, have also been implicated in immune evasion by PCa (10, 11). $CD4^+CD25^{high}$ Treg cells, which are known to control self tolerance in the periphery, derive from the thymus (12, 13) and constitute 1-2% of peripheral lymphocytes in adult humans (14). The importance of these cells in tumor immunity was first demonstrated three decades ago (15). Since then, especially after the introduction of Foxp3

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(16) as a specific marker, studies of numerous mouse tumor models have demonstrated that they can interfere with the anti-tumor immune response at either the induction or effector phase. Increased peripheral and intratumoral Treg densities have been reported in lung, ovarian, colorectal, esophageal and gastric, melanoma, head and neck, prostate and pancreatic cancers (17-20). Evidence for Treg in PCa patients, however, has been limited to a few recent studies with significant controversy (21). Studies from experimental models have demonstrated that Tregs may either be induced or activated within the tumor draining lymph nodes by tumor-derived factors, including tumor-associated antigens (TAA). Then, these cells prevent tumor-specific immune responses either at the induction phase in the tumor draining lymph nodes or at the effector phase within the tumor milieu (22, 23). The first evidence that TAA-specific Tregs may be involved in suppression of tumor-specific immune responses in humans has recently been reported (24). Prostate-Specific Antigen (PSA), a serine protease produced by the prostate gland, is the best known TAA of the prostate. Large amounts of PSA are produced and released into circulation in PCa and BPH patients, as well as in people with prostate inflammation. We demonstrate that the prevalence of Tregs is increased not only in PCa patients, but also in patients with BPH, and that this enhancement is strongly correlated with PSA, suggesting that PSA may have a role in Treg induction and/or maintenance in these people. The varying results concerning peripheral Treg densities in PCa patients and the lack of information regarding a correlation between PSA levels and Treg cell frequency led us to investigate the number of peripheral $CD4^{+}CD25^{high}Foxp3^{+}$ Tregs and their association with total serum PSA levels in PCa and BPH patients. Our results support that the frequency of Tregs increase not only in PCa patients, but also in BPH patients. This enhancement strongly correlates with PSA levels, suggesting a role for PSA in the Treg induction and/or maintenance in these subjects.

TABLE 1. Summary of patient characteristics

Segment	Healthy	BPH	PCa	Total Subjects
Mean Age*	55 (43-68)	60 (44-70)	62 (41-78)	60 (43-78)
PSA*	3.3 (2.5-6)	20.4 (2.7-51)	45.4 (4.8-48)	25.3 (2.5-51)
Number	10	27	19	56

*Numbers in parentheses show range

Methods

Patients and Samples

This project was performed in Akdeniz University immunology laboratory and urology out patient clinic from 2008 to 2010. A total of 56 patients with total serum PSA values of >2.5 ng/ml, who were referred to transrectal ultrasound (TRUS)-guided sextant prostate biopsy at Akdeniz University Urology Outpatient Clinic, were recruited. The mean age of the patients was 62 years (range 41 to 78 years). For Treg screening, 8 ml peripheral blood samples were drawn from patients into heparinized tubes just before the biopsy. None of the patients had any known current infections, and none were taking any immunomodulatory medications and none of them had any cancer before. Blood and biopsy samples were obtained with written consent under an institutional review board-approved protocol. Of the total number of 56 patients that were analyzed, 19 were diagnosed with PCa, 27 with BPH and 10 with no apparent prostate pathology (Table 1). Of Of the 19 PCa patients, 17 had initial stage, locally confined cancer, while 2 had radiologically detected bony metastasis. The Gleason score was used to grade prostate cancer; where a high score is associated with advanced disease and poorer prognosis. Of the 17 locally confined cancer patients, 1 patient scored 5, 5 patients scored 6, 3 patients scored 7 and 10 patients scored 9. Of the 17 with locally confined PCa, 9 were treated by laparoscopic radical prostatectomy at our hospital and were re-analyzed for both total serum PSA and peripheral Treg levels one month following the surgery. 10 who were negative for BPH, PCa and prostatitis through pathological assessment were used as healthy controls. For Treg screening, 8 ml peripheral blood samples were drawn from patients into heparinized tubes just before the biopsy. Blood and biopsy samples

were obtained with written consent under an institutional review board-approved protocol.

Phenotypic and Quantitative Analysis of Lymphocytes:
Peripheral blood mononuclear cells (PBMCs) were isolated using a Fycoll-Hypaque density gradient. Surface staining with anti-human CD4-FITC (BD; 555346), CD25-PE (BD; 555432) and intracellular staining with Foxp3-APC (e-Bioscience; 17-4776-73) antibodies was performed as previously described (22, 25). Briefly, PBMCs were washed three times with D-PBS and stained for surface CD4 and CD25 markers for 30 minutes at room temperature. Finally, the cells were washed twice with saponin buffer and once with washing buffer and analyzed using a BD FACSCalibur Flow Cytometer. Flow-Jo software (Tree Star Inc., San Carlos, CA) was used to analyze the samples and determine the frequencies of Treg cells. Absolute lymphocyte counts were determined by using an automated hematological analyzer (Sysmex XT-2000iV).

Serum PSA Quantification:
Total PSA (free + complexed) from serum samples was measured using an electrochemiluminescence immunoassay (ECLIA) with a Roche Elecsys Modular Analytics E170 immunoassay analyzer according to manufacturer's instructions.

Statistical analysis
All of the statistical analyses were performed using SPSS (version 16; SPSS Inc.). Statistical differences between groups were evaluated by using Kruskas-Wallis analysis. The differences between two groups were determined by the Mann-Whitney test with Benferroni Correction. Correlation was tested by the non-parametric Spearman method. The statistical significance (p value) was set at <0.05.

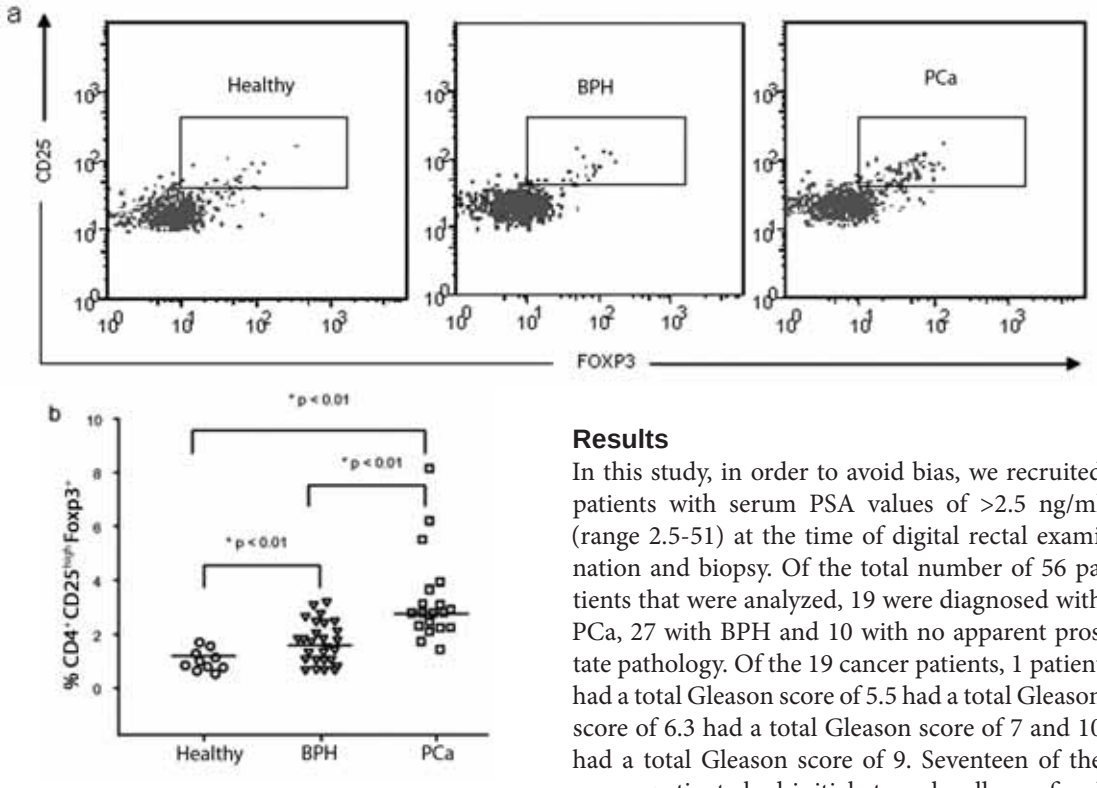

FIGURE 1. (a) The gating strategy used for selecting CD25^{high} cells was very stringent. (b) The mean frequencies of CD4$^+$CD25^{high}Foxp3$^+$ Treg cells as percentages of peripheral lymphocytes were determined for PCa patients, BPH patients and healthy people, respectively.

Results

In this study, in order to avoid bias, we recruited patients with serum PSA values of >2.5 ng/ml (range 2.5-51) at the time of digital rectal examination and biopsy. Of the total number of 56 patients that were analyzed, 19 were diagnosed with PCa, 27 with BPH and 10 with no apparent prostate pathology. Of the 19 cancer patients, 1 patient had a total Gleason score of 5.5 had a total Gleason score of 6.3 had a total Gleason score of 7 and 10 had a total Gleason score of 9. Seventeen of the cancer patients had initial stage, locally confined cancer, while two had radiological detected bone metastasis. Ten people who were negative for BPH, PCa and prostatitis through pathological assessment were used as healthy controls. Peripheral

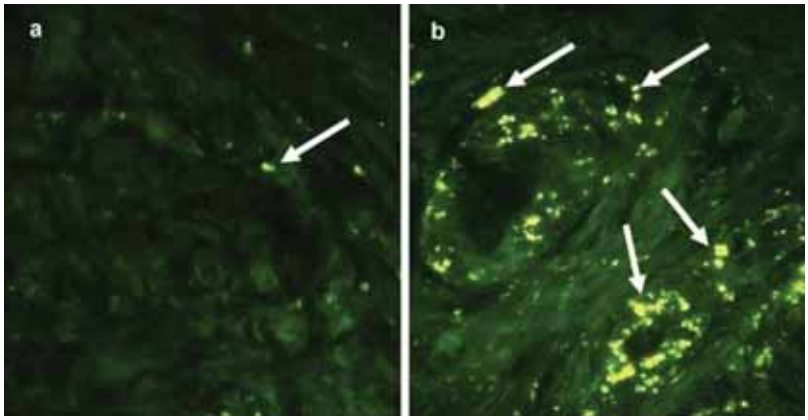

FIGURE 2. (a) Low expressing Treg in Benign tissue (b) Substantial increase in the number of Foxp3 expressing Treg in malignant tissue in Pca patients.

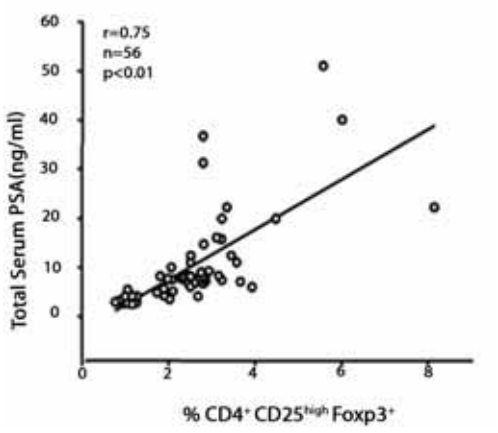

FIGURE 3. Correlation of serum PSA values with peripheral Treg frequencies

blood samples of all the patients at the time of biopsy were analyzed by flow cytometry using CD4, CD25, Foxp3. The gating strategy used for selecting CD25high cells was very stringent (Figure 1a). The mean frequencies of $CD4^+CD25^{high}Foxp3^+$ Treg cells as percentages of peripheral lymphocytes were determined as 3.23% ± 1.59% (n = 19), 1.66% ± 0.80% (n = 27) and 1.08% ± 0.43%, (n = 10) for PCa patients, BPH patients and healthy people, respectively (Figure 1b). Mean frequency of Tregs in PCa patients was significantly higher than that of the BPH patients ($p < 0.01$) and healthy donors ($p < 0.01$). In addition, the mean frequency of Tregs in BPH patients was also significantly higher than that of the healthy donors ($p < 0.01$).

Finally, immunohistochemical staining of prostate tissue sections from 19 PCa patients showed a substantial increase in the number of Foxp3 expressing Treg in malignant tissue (Figure 2b) compared with benign tissue (Figure 2a). There was a strong positive correlation between the two parameters with a correlation coefficient of 0.75 ($p < 0.01$). Nine PCa patients, who were treated by laparoscopic radical prostatectomy, were re-analyzed one-month post surgery for peripheral PSA and Treg levels (Figure 3). The mean level of total serum PSA was 10.00 ± 5.97 and the mean frequency of peripheral Treg was 3.20±1.61 before the surgery. After curative prostatectomy, serum PSA levels of all the patients were reduced to very low/undetectable levels as expected [(0.20±0.49; paired t-test $p < 0.01$) (Figure 4a, b)]. Strikingly, the mean Treg frequencies in these patients also decreased significantly [(1.09 ± 0.32; paired t-test $p < 0.01$) (Figure 4c, d)]. This result suggests that PSA alone or in combination with other tumor derived factors may be required for the increased presence of Treg in the periphery. In order to rule out a possible post-surgery stress effect on Treg frequencies, we recruited six more patients, in addition to our study group described above, that had previously planned to undergo non-PCa related prostate surgeries at our hospital. These people were screened for Treg frequencies before and one month after the surgery. In these patients, the frequencies of Treg did not change significantly after the surgery (1.11±0.20 to 1.13±0.22; paired t-test $p > 0.05$), demonstrating that surgery by itself does not cause a decrease in Treg frequency (Figure 4e, f).

Discussion

The purpose of this study was two-fold. First, we sought to verify whether the peripheral frequencies of $CD4^+CD25^{high}Foxp3^+$ Treg cells are elevated

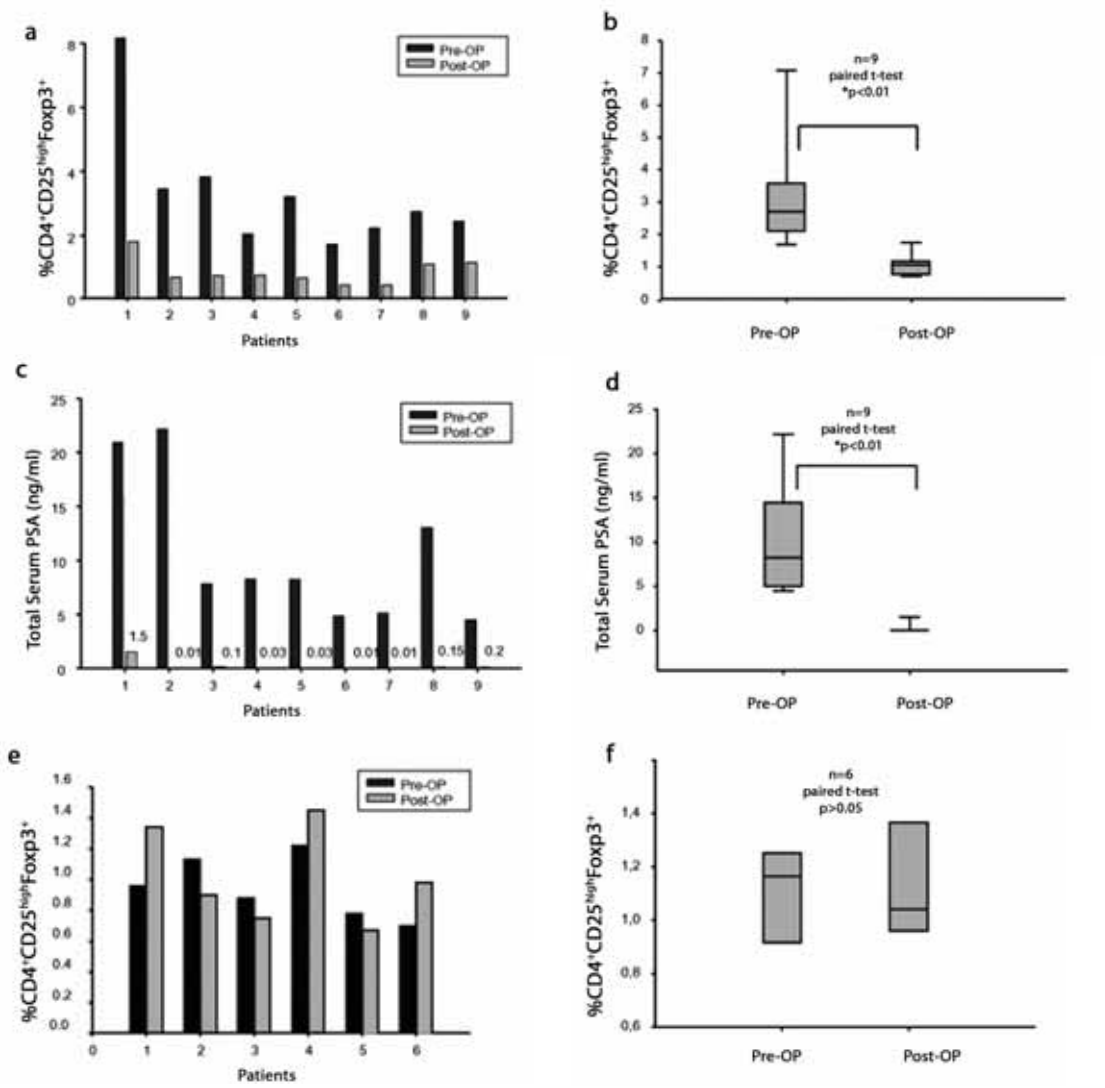

FIGURE 4. (a) Treg frequencies of individual patients before and after the surgery. (b) Mean Treg frequencies (3.20 ± 1.61) were significantly decreased after the surgery (1.09 ± 0.32). (c) Total serum PSA levels of individual patients before and after the surgery. (d) Mean serum PSA levels (10.00 ± 5.97) were significantly decreased after the surgery (0.20 ± 0.49). (e) Treg frequencies of individual patients before and after non-PCa related surgery are shown. (f) Mean Treg frequencies (1.11 ± 0.20) were not significantly changed after the non- PCa related surgery (1.13 ± 0.27).

in PCa patients; second, we aimed to determine the direct correlation between the peripheral Treg and total serum PSA levels in these patients. We first demonstrated that $CD4^{+}CD25^{high}Foxp3^{+}$ Treg densities are increased in PCa patients compared with BPH patients and healthy controls. In addition to this observation, which is consistent with the findings of Miller et al., (10) we also recorded a significant enhancement in Treg frequency in the periphery of BPH patients compared with healthy controls. Even though Miller et al., (10) also clearly showed increased levels of Treg infiltration in prostate tissue samples of BPH patients, these authors did not analyze those patients for peripheral Treg densities. Our results and those of Miller et al., however, contradict findings of Yokokawa et al.(11), who observed no change in the frequencies of Treg cells in PCa patients except

in those with metastatic cancers. Seventeen of 19 patients in the current study and all of the patients by Miller et al.(10) were reported to have locally confined cancer. Furthermore, despite the fact that the mean frequency of Tregs in PCa patients were comparable in all three studies, the mean frequency reported for healthy subjects by Yokokawa et al.(11) was considerably higher than the recorded results from our study as well as others, including Miller et al.(10) This discrepancy might have resulted from the age of healthy controls used, which was 55 years (range 41-78 years) in our study. Furthermore, our analysis clearly demonstrates that Tregs from PCa patients have significant suppressive activity. Secondly and more importantly, we demonstrate for the first time that a strong association exists between the high density of Tregs and total serum PSA in both BPH and PCa patients, suggesting that the increased frequency of Tregs may not be a result of malignity, but may rather be caused by the excessive amounts of PSA accumulation in these patients. We obtained the first line of evidence for this assumption during the diagnostic process, by showing that Treg frequencies were strongly correlated with serum PSA in patients with serum PSA levels of 2.5-51 ng/ml, regardless of the disease status ($r = 0.75$, $p < 0.05$). The evidence for the strong association of PSA with Tregs was obtained from the analysis of nine patients with locally confined cancers, who were treated by radical prostatectomy. Treg frequencies of all these patients subsided remarkably one month following surgery. Interestingly, although three of these patients had Treg levels near the threshold Treg frequency of 2.1% (95% CI), these levels further declined. This suggests that tumor-specific Tregs might still exist and be depleted upon antigen removal, even in patients whose Treg levels are not elevated. Our result is in agreement with Kono et al. (30), who have recently observed a similar reduction of peripheral Treg levels in gastric cancer patients that underwent curative surgeries. Taken together, our results strongly suggest that PSA, either alone or with other prostate derived factors, is involved in either the induction and/or maintenance of peripheral Tregs in BPH and PCa patients. This study was limited by patients number, because our clinic is not a urologic cancer clinic. The reason for the enhanced number of Tregs in human cancers is not clear. It is hypothesized that TAA in the presence of soluble mediators, such as TGF-β and chemokines may be required for peripheral induction and/or expansion of Tregs within the tumor-draining lymph nodes. We also do not know whether other factors, either tumor-derived or tumor-induced, are also required in these processes. Previous *in vitro* studies demonstrated that PSA is able to induce TGF-β production and impair dendritic cell maturation. Both of these pathways are known to induce Tregs, *in vivo* in experimental models and *in vitro* in human PBMC cultures and thus, it is reasonable to assume that excessive amounts of PSA in both BPH and PCa patients may invoke one or both of the above pathways to either induce or expand PSA-specific Tregs. It is also likely that other mechanisms, such as IDO (Indoleamin 2-3 deoxygenase) or PGE-2 production by the cells within the tumor milieu might contribute to the process. Further studies addressing these questions will be important for our understanding of the biology of the Treg in human cancer. Restoring peripheral Treg levels upon removal of cancerous prostates also has important implications for immunotherapy for PCa. Many prostate-specific vaccine trials using PSA peptides to stimulate tumor-specific immune responses before the surgical removal of tumors have not yielded desired results.

Conclusions

In this study we present, to our knowledge, the first evidence in the literature that increased frequency of circulating $CD4^{+}CD25^{high}Foxp3^{+}$ Tregs in PCa and BPH patients is correlated with PSA levels and also the first to demonstrate a strong dynamic association between a TAA and Tregs in cancer bearing humans. Our results demonstrate that peripheral Treg densities are correlated with PSA in BPH and PCa patients, suggesting that PSA may have a role in Treg induction and/or maintenance in Treg in these people. In the light of these findings, we may expect better response of treatment of tumour specific immunotherapy after removal of prostate gland which includes adenocarcinoma cells. More comprehensive studies on this issue by holding the light of findings in immunotherapy for PCa may provide new forms of treatments.

Competing interests

The authors declare that we have no financial and personal relationships with other people or organizations that could inappropriately influence this work.

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